

Unique targeting of cytosolic phospholipase A₂ to plasma membranes mediated by the NADPH oxidase in phagocytes

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Cytosolic phospholipase A₂ (cPLA₂)-generated arachidonic acid (AA) has been shown to be an essential requirement for the activation of NADPH oxidase, in addition to its being the major enzyme involved in the formation of eicosanoid at the nuclear membranes. The mechanism by which cPLA₂ regulates NADPH oxidase activity is not known, particularly since the NADPH oxidase complex is localized in the plasma membranes of stimulated cells. The present study is the first to demonstrate that upon stimulation cPLA₂ is transiently recruited to the plasma membranes by a functional NADPH oxidase in neutrophils and in granulocyte-like PLB-985 cells. Coimmunoprecipitation experiments and double labeling immunofluorescence

analysis demonstrated the unique colocalization of cPLA₂ and the NADPH oxidase in plasma membranes of stimulated cells, in correlation with the kinetic burst of superoxide production. A specific affinity *in vitro* binding was detected between GST-p47^{phox} or GST-p67^{phox} and cPLA₂ in lysates of stimulated cells. The association between these two enzymes provides the molecular basis for AA released by cPLA₂ to activate the assembled NADPH oxidase. The ability of cPLA₂ to regulate two different functions in the same cells (superoxide generation and eicosanoid production) is achieved by a novel dual subcellular localization of cPLA₂ to different targets.

Introduction

The NADPH oxidase is a multicomponent electron transport chain that transfers electrons from NADPH to molecular oxygen to form superoxide, a precursor of microbicidal oxidants. The production of superoxide by NADPH oxidase is one of the most important functions for host defense. However, during altered physiological states reactive oxygen products may promote inflammatory reactions and participate in processes that lead to tissue injury. An understanding of the biochemical processes that regulate NADPH oxidase activity may provide a means to more effectively control the activity of the cells during infection and inflammation. In resting neutrophils, NADPH oxidase is dormant, with unassembled subunits located on the cytosol and the

plasma membranes (Babior, 1999; Leto, 1999; Babior et al., 2002). Upon stimulation, the cytosolic components p47^{phox}, p67^{phox}, p40^{phox}, and rac2 translocate to the plasma membranes and associate with the heterodimeric transmembrane glycoprotein, flavocytochrome b₅₅₈. The cytochrome is comprised of two subunits, gp91^{phox} and p22^{phox}, that contain heme, flavin, and NADPH-binding sites (Segal and Abo, 1993; Babior, 1999; Leto, 1999; Yu et al., 1999; Segal et al., 2000). The two cytosolic components, p47^{phox} and p67^{phox}, contain Src homology 3 motifs that direct their translocation to the membranes by binding to specific targets in p22^{phox} and to each other (Leto, 1999). p47^{phox} and p40^{phox} subunits contain phox homology domains that specifically bind to phosphatidylinositol 3,4-bisphosphate and phosphatidylinositol 3-phosphate (Ellson et al., 2001; Kanai et al., 2001; Sato et al., 2001). These phox homol-

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Abbreviations used in this paper: AA, arachidonic acid; cPLA₂, cytosolic phospholipase A₂; fMLP, formyl-methionyl-leucyl-phenylalanine; OZ, opsonized zymosan; PtdIns(4,5)P₂, phosphatidylinositol 4,5-bisphosphate.

ogy domains are thought to participate in directing the subunits to the membranes and in assembling the oxidase complex (Ponting, 1996; Kanai et al., 2001; Stahelin et al., 2003a).

Cytosolic phospholipase A₂ (cPLA₂) which hydrolyzes phospholipids containing arachidonate at the sn-2 position (Clark et al., 1990; Kramer et al., 1991), has been implicated as the major enzyme in the formation of proinflammatory lipid mediators. Recently, we have created in the human phagocyte myeloid cell line, PLB-985, a p85 cPLA₂-deficient model cell (Dana et al., 1998) and demonstrated an essential requirement for arachidonic acid (AA) in activation of the assembled phagocyte NADPH oxidase, the oxidase-associated H⁺ channel (Lowenthal and Levy, 1999), and the oxidase-associated diaphorase activity (Pessach et al., 2001) induced with a variety of agonists. The normal translocation of the oxidase cytosolic components in activated differentiated PLB-985 cells lacking cPLA₂ or in stimulated neutrophils in the presence of PLA₂ inhibitors (Levy et al., 1994; Dana et al., 1998; Pessach et al., 2001) and the effect of PLA₂ inhibitors even when added after oxidase activation (Henderson et al., 1993) indicate that cPLA₂ is not required for translocation of the cytosolic factors to the membranes but rather serves a critical role in oxidase activation after the assembly of the oxidase complex. Addition of free AA but not other free fatty acids nor AA metabolites restored the activity, indicating the specificity of AA for the process. The requirement of cPLA₂ for oxidase activation is consistent with our and other previous studies in human neutrophils and monocytes (Henderson et al., 1993; Dana et al., 1994; Li and Cathcart, 1997; Bae et al., 2000). In addition, a recent study (Zhao et al., 2002) has demonstrated by the use of antisense molecules that cPLA₂ is absolutely required for superoxide generation in monocytes stimulated with opsonized zymosan. However, that study suggests that cPLA₂ is required for translocation of oxidase cytosolic components to the membranes and not to activation of the assembled oxidase. The discrepancy may be due to the different roles of cPLA₂ in the different cell types, as proposed by the authors. In contrast to the findings demonstrating requirement of the cPLA₂ for oxidase activation, resident peritoneal macrophages from cPLA₂-deficient mice exhibited normal stimulated superoxide release (Gijon et al., 2000), which may be attributed to either compensatory effects of isoenzymes frequently observed in models of knockout animals or to the known differences of the PLA₂ isotypes between mice and human/rat (Suzuki et al., 2000). In an earlier study, we showed that AA increases the affinity of the assembled oxidase in purified membranes for NADPH (Rubinek and Levy, 1993). The mechanism by which cPLA₂ regulates the NADPH oxidase activity is not known, particularly since the NADPH oxidase complex is localized in the plasma membrane in stimulated cells, whereas cPLA₂ has been shown to translocate to the nuclear and ER membranes in a variety of cells (for review see Hirabayashi and Shimizu, 2000). To elucidate the mechanism by which cPLA₂ regulates the activation of NADPH oxidase, the present study investigated the cellular localization of cPLA₂ upon stimulation and whether

there is any association between cPLA₂ and the NADPH oxidase complex during activation of phagocytic cells.

Results

Coimmunoprecipitation of cPLA₂ with NADPH oxidase in membranes of activated neutrophils

The association between cPLA₂ and NADPH oxidase was studied by performing coimmunoprecipitation experiments in resting and stimulated neutrophils with either 1 mg/ml opsonized zymosan (OZ), 50 ng/ml PMA, or 5 × 10⁻⁷ M formyl-methionyl-leucyl-phenylalanine (fMLP). As shown in Fig. 1, addition of antibodies against either p47^{phox} or p67^{phox} resulted in significant immunoprecipitation of the cytosolic components in the membrane fractions of the activated neutrophils. cPLA₂ was coimmunoprecipitated with either of the cytosolic components (p47^{phox} or p67^{phox}) in the membrane fractions of activated neutrophils but not in the membranes of resting neutrophils. In spite of the high levels of p47^{phox} or p67^{phox} immunoprecipitated in the cytosol of resting or activated neutrophils, coimmunoprecipitation of cPLA₂ was not detected. To confirm the binding between cPLA₂ and the NADPH oxidase complex, cPLA₂ was immunoprecipitated, and its association with oxidase components was analyzed. As shown in Fig. 2 A, cPLA₂ was immunoprecipitated in the membrane fractions of neutrophils activated for 1 min with either OZ, fMLP, or PMA. The NADPH oxidase components, p47^{phox}, p67^{phox}, and gp91^{phox}, were coimmunoprecipitated with cPLA₂ in the membrane fractions of activated cells. Traces of cPLA₂ and the oxidase components detected in membranes of resting cells (Figs. 1 and 2) are probably due to some basal stimulation of neutrophils during purification. When cPLA₂ immunoprecipitation was done with preimmune serum, neither cPLA₂ nor the oxidase components were detected (unpublished data), confirming the specificity of the assay.

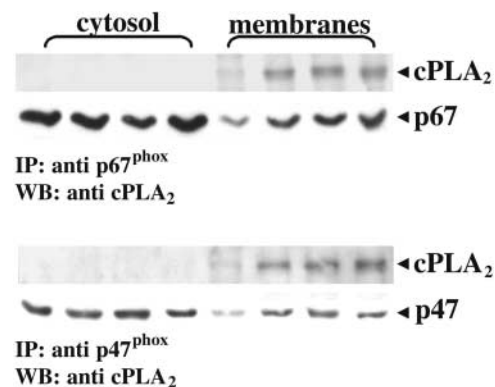


Figure 1. Coimmunoprecipitation of cPLA₂ with p67^{phox} or p47^{phox} in membranes of stimulated neutrophils. Neutrophils were stimulated with 1 mg/ml OZ, 5 × 10⁻⁷ M fMLP, or 50 ng/ml PMA for 1 min at 37°C. The cell cytosol and solubilized membranes were subjected to immunoprecipitation (IP) with anti-p67^{phox} or anti-p47^{phox} antibodies. The immunoprecipitates were separated by SDS-PAGE electrophoresis, and immunoblotting was performed with anti-cPLA₂ antibodies. The levels of p67^{phox} or p47^{phox} immunoprecipitated were evaluated by immunoblot analysis. The results shown are from one representative experiment out of three that gave identical results.

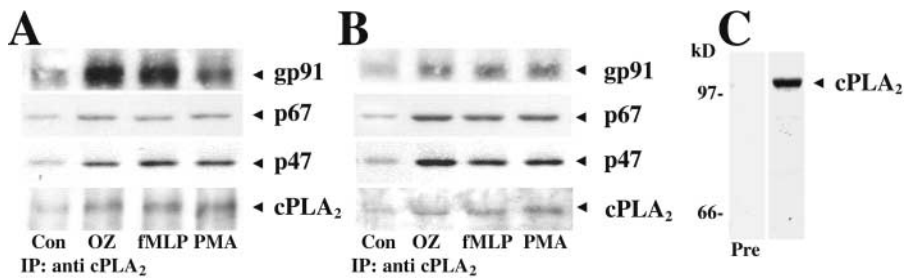


Figure 2. Coimmunoprecipitation of NADPH oxidase components with cPLA₂ in membranes of activated neutrophils. Neutrophils (A) and neutrophils (B) preincubated with 5 μ g/ml cytochalasin b for 10 min at 37°C were stimulated as in the legend to Fig. 1. cPLA₂ immunoprecipitation (IP) was performed in solubilized neutrophil membranes, applied to SDS-PAGE electrophoresis, and followed by immunoblotting with

anti-gp91^{phox}, anti-p67^{phox}, and anti-p47^{phox} antibodies. The levels of cPLA₂ immunoprecipitated were evaluated by immunoblot analysis. The results shown are from one representative experiment out of three that gave identical results. (C) Immunoblot of cPLA₂ in neutrophil lysates. Preimmune serum (pre) did not detect any protein, whereas the antibodies raised against cPLA₂ detected one band in the neutrophil lysates.

Pretreatment of neutrophils with 5 μ g/ml cytochalasin b, which prevents actin polymerization and disrupts cytoskeleton integrity (Frank, 1990; Tsai et al., 1994; Sheikh and Nash, 1998), did not affect the binding between cPLA₂ and the assembled oxidase in membranes of the stimulated neutrophils (Fig. 2 B). Although several studies have suggested that the functional oxidase complex at the plasma membranes is associated with the cytoskeleton (Nauseef et al., 1991; Dusi et al., 1996; Allen et al., 1999), the present findings suggest that assembly of the NADPH oxidase complex and its association with cPLA₂ stimulated by these agonists are independent of an intact cytoskeleton in nonadherent neutrophils. Furthermore, in accordance with these results superoxide generation and translocation of the cytosolic oxidase components to membranes of nonadherent neutrophils pretreated with cytochalasin b was increased when stimulated with fMLP or OZ but was not affected when stimulated with PMA (unpublished data; Dusi et al., 1996; Yan and Novak, 1999; Jiang et al., 2000).

Translocation of cPLA₂ to the plasma membranes of activated neutrophils

To determine the exact subcellular localization of cPLA₂ after neutrophil activation, resting and stimulated cells were fractionated onto a three-step discontinuous Percoll gradient, resulting in the separation of azurophil granules (α), the specific granules (β_1 and β_2), and the plasma membranes/secretory vesicles (γ). Fig. 3 demonstrates that both cPLA₂ and p47^{phox} were associated with the γ fraction of PMA-stimulated neutrophils as shown earlier for p47^{phox} (el Benna et al., 1994; McAdara Berkowitz et al., 2001). Neutrophil

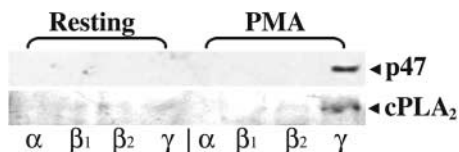


Figure 3. Subcellular localization of cPLA₂ in neutrophils. 5×10^8 neutrophils, either unperturbed or stimulated with PMA, were disrupted by N₂ cavitation. The postnuclear supernatant was loaded on a three-layer Percoll gradient. The α , β_1 , β_2 , and γ fractions were formed as described in Materials and methods, separated on SDS-PAGE electrophoresis, and subjected to immunoblot analysis with anti-p47^{phox} and anti-cPLA₂ antibodies. The results are from one representative experiment out of three that gave identical results.

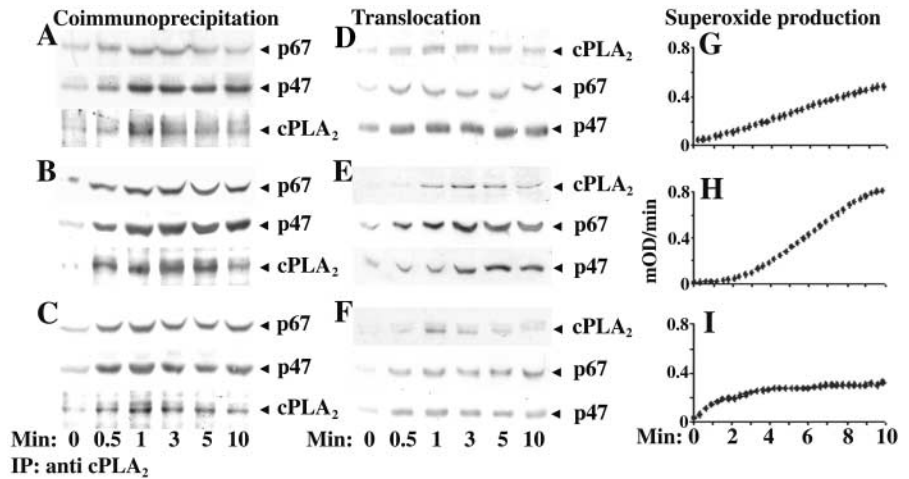
stimulation with either fMLP or OZ produced the same results (unpublished data).

Time course binding between cPLA₂ and the assembled NADPH oxidase

The binding kinetics between cPLA₂ and the assembled NADPH oxidase were studied in peripheral blood neutrophils and in granulocyte-like PLB-985 cells and revealed identical results, as shown for granulocyte-like PLB-985 cells in Fig. 4. Immunoprecipitation of cPLA₂ from solubilized membrane fractions could be detected as early as 30 s after stimulation, reaching maximal levels after 1 min of stimulation with either 1 mg/ml OZ or 5×10^{-7} M fMLP and decreasing thereafter (Fig. 4, A and C). Similar results were obtained when the cells were stimulated with 50 ng/ml PMA, with maximal levels after 3 min of stimulation (Fig. 4 B). Since cPLA₂ was immunoprecipitated from the membranes, its level dictated the levels of coimmunoprecipitated p47^{phox} and p67^{phox} (Fig. 4, A–C). Thus, the time course appearance of cPLA₂, p47^{phox}, and p67^{phox} to the membranes after stimulation was analyzed in order to compare translocation kinetics of these proteins. As shown in Fig. 4, D–F, the oxidase cytosolic components were rapidly and simultaneously translocated to the solubilized membrane fractions after stimulation, preceding the translocation of cPLA₂. These results are in line with our previous studies (Levy et al., 1994; Dana et al., 1998; Pessach et al., 2001), demonstrating that the translocation of the oxidase cytosolic components is independent of cPLA₂. As shown in Fig. 4, D–F, the kinetics of cPLA₂ translocation to the membranes is dependent on the translocation of the kinetics of oxidase cytosolic components and is more rapid when stimulated with OZ or fMLP than with PMA, which is consistent with the development of the burst of superoxides by these agonists (Fig. 4, G–I). Significant translocation of cPLA₂ to the membranes of stimulated cells was detected before the production of superoxides, whereas maximal translocation of cPLA₂ was detected at 1 min of stimulation with OZ or fMLP and at 3 min of stimulation with PMA (Fig. 4, D–F), which coincided with the onset of superoxide production by these three agonists (Fig. 4, G–I). The detachment of cPLA₂ from the membrane fractions precedes that of the cytosolic components p47^{phox} and p67^{phox} (Fig. 4, D–F) and takes place while NADPH oxidase is still being activated to generate superoxide (Fig. 4, G–I).

To determine the molar ratio between the assembled oxidase and cPLA₂ in the membranes after stimulation, the lev-

Figure 4. The time course association and translocation of cPLA₂ and the cytosolic components of NADPH oxidase in membranes of stimulated granulocyte-like PLB-985 cells. PLB-985 cells were differentiated with 1.25% DMSO for 4 d. The cells were stimulated with 1 mg/ml OZ (A), 50 ng/ml PMA (B), or 5×10^{-7} M fMLP (C) for the indicated times at 37°C. The cell membranes were subjected to immunoprecipitation (IP) with anti-cPLA₂ antibodies, followed by immunoblot of p67^{phox} and p47^{phox}. The bottom lane in each experiment shows the levels of cPLA₂ detected in the immunoprecipitates. Time course translocation of cPLA₂, p67^{phox}, and p47^{phox} to cell membranes after stimulation with OZ (D) PMA (E), or fMLP (F) was detected by immunoblot analysis. 2×10^6 cell membrane equivalent were applied per lane. Superoxide production stimulated with either OZ (G), PMA (H), or fMLP (I) as detected by cytochrome C reduction. The results are from one representative experiment out of three presenting identical results.



els of cPLA₂ and of p47^{phox} (which are representative of the assembled oxidase) in the same membranes from stimulated neutrophils were analyzed by quantitative Western blotting as done by others (Quinn et al., 1993). Representative blots and standard curves obtained by quantitative densitometry of recombinant proteins are shown in Fig. 5. Quantitative analysis revealed that cPLA₂ and p47^{phox} translocated to the membranes by the three agonists at a molar ratio of 1:25. Actual measured values for cPLA₂ derived from the standard curves were 2.1, 1.6, or 2.3 ng/sample, which were calculated as 25, 20, or 28 pmoles/sample in the membranes from neutrophils stimulated with OZ, fMLP, or PMA, respectively. Actual measured values for p47^{phox} derived from the standard curves were 28, 23, or 31 ng/sample, which were calculated as 604, 504, or 673 pmoles/sample in the membranes from cells stimulated with OZ, fMLP, or PMA, respectively. Despite the different levels of p47^{phox} translocated to the membrane induced by the different agonists, the molar ratio between cPLA₂ and p47^{phox} was similar, indicating that the level of p47^{phox} determines the level of cPLA₂ that translocates to the membranes. Similar results were obtained in granulocyte-like PLB-985 cells.

In vivo location of cPLA₂ in resting and activated cells

Based on the in vitro binding between cPLA₂ and NADPH oxidase in plasma membranes of stimulated cells, we examined the in vivo location of cPLA₂ in resting and activated cells by immunofluorescence microscopy. Granulocyte-like PLB-985 cells are advantageous for the study: first, the expression of gp91^{phox} can be manipulated in these cells (Zhen et al., 1993), thus enabling determination of the role of oxidase for targeting cPLA₂ to the plasma membranes, and second, PLB-985 cells differentiated with DMSO exhibit different kinetics for superoxide production (Fig. 4) and for eicosonoid formation (shown at the end of this section), thus facilitating a means to detect the location of cPLA₂ in different compartments after stimulation. Double staining immunofluorescence analysis of cPLA₂ and the membrane oxidase component gp91^{phox} showed that cPLA₂ is found in the cytosol of resting granulocyte-like PLB-985 cells (Fig. 6 A). Upon stim-

ulation with either PMA, OZ, or fMLP, a significant translocation of cPLA₂ to the cell periphery was detected where it colocalized with gp91^{phox}, confirming the localization of cPLA₂ in the plasma membranes of activated phagocytic cells. No increase in the gp91^{phox} immunofluorescence signal was detected after stimulation with either of the agonists (Fig. 6 A), which is in line with the low levels of granules, including gp91^{phox} endomembranes, present in PLB-985 cells differenti-

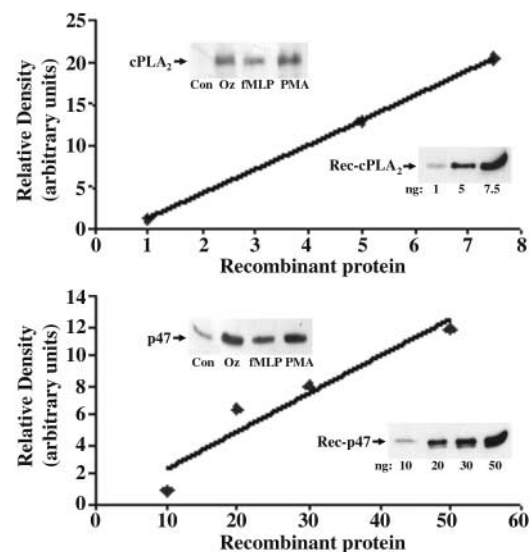


Figure 5. The molar ratio between cPLA₂ and NADPH oxidase in the membranes of stimulated cells. Samples of recombinant cPLA₂ or p47^{phox} and membranes (bottom inset) of neutrophils stimulated with 1 mg/ml OZ, 5×10^{-7} M fMLP for 1 min or 50 ng/ml PMA for 3 min (top inset) were analyzed by SDS-PAGE electrophoresis. 2×10^6 cell membrane equivalents were applied per lane. The Western blots were analyzed using quantitative densitometry, and the relative optical density of each band was plotted against the amount of protein added per lane. The correlation coefficient for the cPLA₂ standard curve is 0.99 and for the p47^{phox} standard curve is 0.92. The quantities of cPLA₂ and p47^{phox} in membranes of stimulated cells were determined from the standard curves. The results are from one representative experiment out of three presenting identical results.

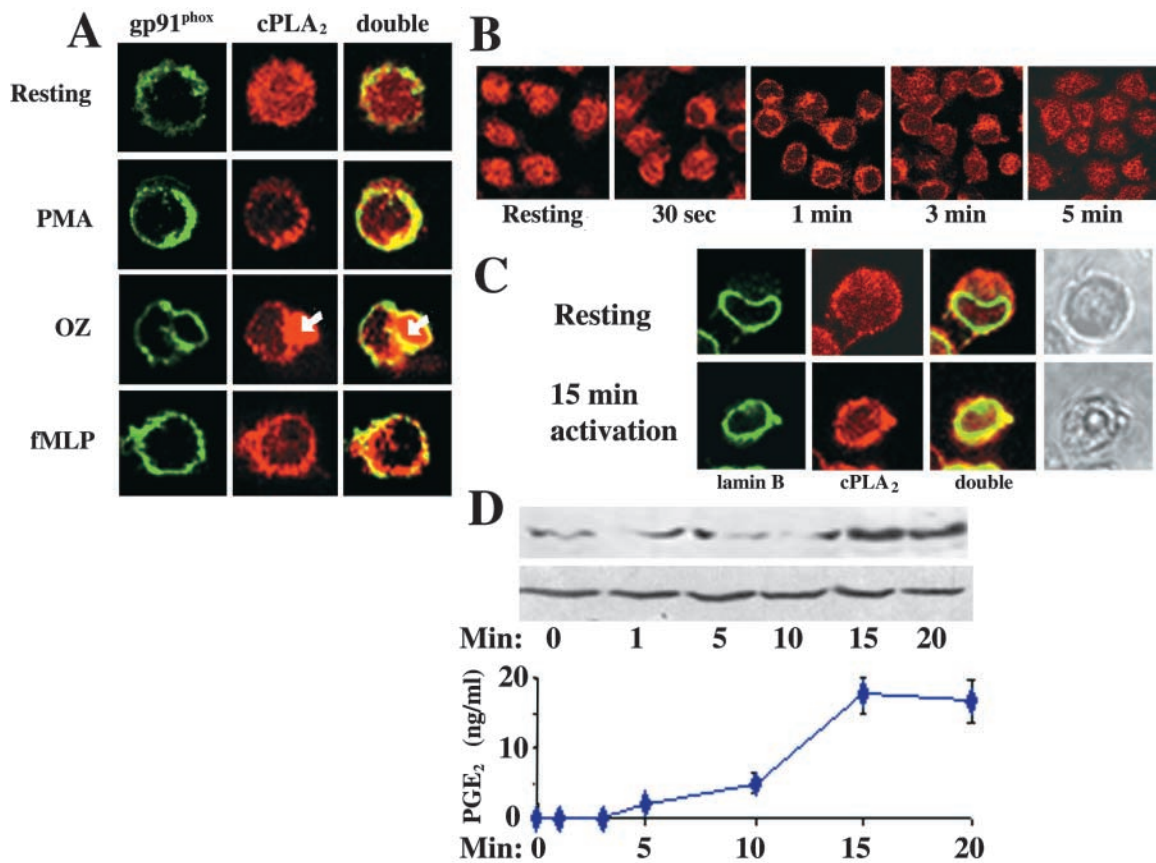


Figure 6. Subcellular localization of cPLA₂ in stimulated granulocyte-like PLB-985 cells. (A) Granulocyte-like PLB-985 cells before and after stimulation with 1 mg/ml OZ, 5 × 10⁻⁷ M fMLP for 1 min or 50 ng/ml PMA for 3 min at 37°C were fixed, permeabilized, and incubated with anti-cPLA₂ and anti-gp91^{phox} antibodies and then with cy3- and cy2-conjugated second antibodies, respectively. cPLA₂ was found in the cytosol in resting cells and colocalized with gp91^{phox} in the plasma membranes in stimulated cells. An engulfed OZ particle (stained in red as indicated by the white arrow) is surrounded by plasma membranes in which gp91^{phox} and cPLA₂ colocalized (×1,000). (B) The time course translocation of cPLA₂ to cell periphery after stimulation with 5 × 10⁻⁷ M fMLP (×400). (C) Lamin B was labeled by anti-lamin B antibodies and then with cy2-conjugated second antibodies. cPLA₂ colocalized with lamin B in nuclear membranes of stimulated cells with 5 × 10⁻⁷ M fMLP for 15 min (×1,000). (D) The kinetics ± SEM of PGE₂ production (of three experiments, each in triplicates) and of cPLA₂ translocation to the nuclear fractions (a representative immunoblot analysis of the three experiments) in 5 × 10⁶ cells/ml granulocyte-like PLB-985 cells after stimulation with 5 × 10⁻⁷ M fMLP. 2 × 10⁶ cell equivalent of nuclear fraction was applied in each lane. Equal amounts of the nuclear fractions were confirmed by detection of the levels of lamin B in each sample by immunoblot analysis.

ated with DMSO but in contrast to PLB-985 cells differentiated with 1,25(OH)₂D₃ (unpublished data) or to PLB-985 cells differentiated with DMF (Pedruzzi et al., 2002). The kinetics of cPLA₂ translocation stimulated with fMLP is depicted in Fig. 6 B. Maximal levels of cPLA₂ were detected in the cell periphery 1 min after stimulation. 5 min after stimulation, a significant fraction of cPLA₂ reappeared in the cytosol. This subcellular localization of cPLA₂ stimulated with fMLP is similar to that detected by immunoblotting analysis (Fig. 4 D). The immediate translocation of cPLA₂ to the membranes correlates with the rapid onset of superoxide upon stimulation (Fig. 4). Translocation of cPLA₂ to the nucleus was studied by double labeling of cPLA₂ and of lamin B as a marker for nuclear membranes (Olins et al., 2001). As shown in Fig. 6 C, activation of the cells with fMLP for 15 min induced translocation of cPLA₂ to the nuclear membranes where it colocalized with lamin B. To determine the kinetics of cPLA₂ translocation to the nuclear membranes, the nuclei of cells stimulated for different time duration were separated and analyzed for the presence of cPLA₂ by immunoblot

analyses. Translocation of cPLA₂ to the nuclear membranes could be detected only 15 min after stimulation, in correlation with the time course of PGE₂ secretion as shown in Fig. 6 D, which depicts stimulation of granulocyte-like PLB cells with fMLP. Similar results were obtained with PMA and OZ (unpublished data).

The role of NADPH oxidase in targeting cPLA₂ to the plasma membranes

To determine the role of NADPH oxidase in directing cPLA₂ to the plasma membranes, we studied the subcellular location of cPLA₂ in granulocyte-like gp91^{phox}-deficient PLB-985 cells in which no oxidase assembly occurs (Bibershteyn-Kinkade et al., 1999). As shown by immunoblot analysis, cPLA₂ could not be detected in membranes of granulocyte-like gp91^{phox}-deficient PLB-985 cells stimulated for 1 min with OZ, PMA, or fMLP, whereas it was clearly detected in membrane fractions of stimulated granulocyte-like PLB-985 cells (Fig. 7 A). These results were confirmed by

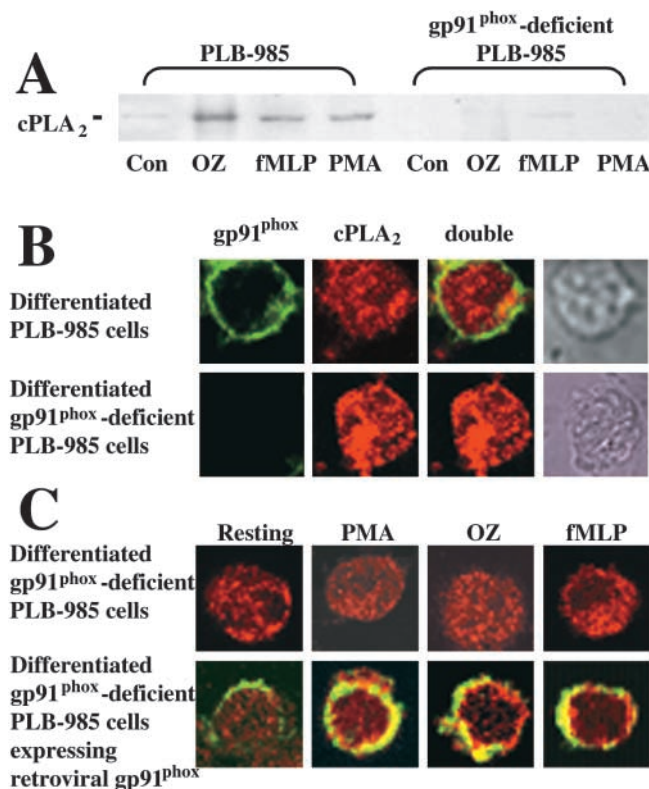


Figure 7. Absence of gp91^{phox} in the cell membrane prevents cPLA₂ translocation to the plasma membranes. (A) Granulocyte-like PLB-985 and granulocyte-like gp91^{phox}-deficient PLB-985 cells were stimulated as in the legend to Fig. 1. The cell membranes were separated on 10% SDS-PAGE and subjected to Western blot analysis against cPLA₂. The results are from one representative experiment out of three that gave identical results. (B) Resting granulocyte-like and granulocyte-like gp91^{phox}-deficient PLB-985 cells were double stained as in the legend to Fig. 6 A. cPLA₂ is found in the cytosol of both type of cells, whereas gp91^{phox} was detected only in granulocyte-like PLB-985 cells. (C) Granulocyte-like gp91^{phox}-deficient PLB-985 cells and the cell line expressing retroviral gp91^{phox} differentiated toward the granulocytic lineage before and after stimulation (as in the legend to Fig. 1) were double stained (as in the legend to Fig. 6 A). cPLA₂ is found in the cytosol before and after activation in gp91^{phox}-deficient PLB-985 cells but translocates to cell periphery after stimulation following the expression of retroviral gp91^{phox} protein in these cells.

confocal laser scanning microscopy, demonstrating that granulocyte-like gp91^{phox}-deficient PLB-985 cells indeed do not express any gp91^{phox} (Fig. 7 B) and that their stimulation with either of the three agonists does not induce any translocation of cPLA₂ to the cell periphery (Fig. 7 C). However, after expression of retroviral gp91^{phox} protein in these cells, cPLA₂ translocation to the cell periphery was fully restored (Fig. 7 C), thereby establishing the role of a functional oxidase for targeting cPLA₂ to the plasma membranes.

In vitro interaction between cytosolic oxidase components and cPLA₂

To determine affinity binding between cPLA₂ and the oxidase components, GST-p47^{phox} and GST-p67^{phox} fusion proteins were used. As shown in Fig. 8, cPLA₂ was pulled down from lysates of stimulated, but not resting, neutrophils

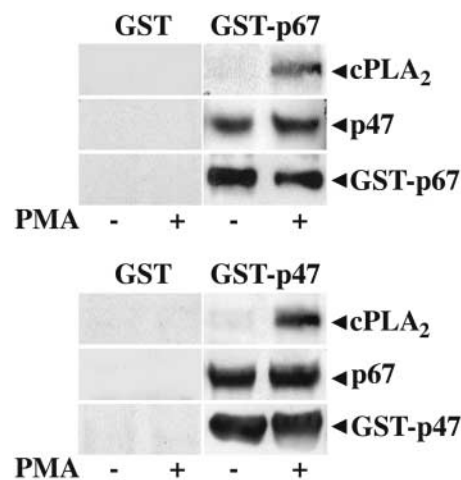


Figure 8. Binding between cPLA₂ and cytosolic oxidase components. Affinity-binding assay using GST-p67^{phox} or GST-p47^{phox} was performed as described in Materials and methods. GST-p67^{phox} binds p47^{phox} in lysates of resting and stimulated neutrophils (with 50 ng/ml PMA) and binds cPLA₂ only in lysates of stimulated cells, whereas GST alone was not effective. Similarly, GST-p47^{phox} binds p67^{phox} in lysates of resting and stimulated cells and binds cPLA₂ only in lysates of stimulated cells.

by either GST-p47^{phox} or GST-p67^{phox} fusion protein but not from lysates of resting cells, suggesting that the binding occurs only with the phosphorylated form of cPLA₂. In addition, p67^{phox} was also pulled down by GST-p47^{phox} fusion protein, and p47^{phox} was pulled down by GST-p67^{phox} fusion protein. These experiments demonstrate the in vitro affinity binding between cPLA₂ and the oxidase cytosolic components but do not determine whether cPLA₂ is associated independently with both p47^{phox} and p67^{phox} or whether it is bound to one cytosolic component and takes up the other, since p47^{phox} and p67^{phox} are found in a complex at the membranes of activated cells. Similar results were obtained in granulocyte-like PLB-985 cells (unpublished data). Although cPLA₂ was specifically bound to each of the oxidase cytosolic components in vitro using recombinant GST fusion proteins, such binding between cPLA₂ and the soluble p47^{phox} and p67^{phox} in the cytosol did not occur in physiological conditions (Fig. 1). Rather cPLA₂ binds the cytosolic oxidase components when found in the assembled NADPH oxidase after translocation to the plasma membranes, in accordance with our previous study demonstrating that the assembly of the oxidase is independent of cPLA₂ (Dana et al., 1998). The behavior of the recombinant GST-fused cytosolic proteins may reflect the effects of the fusion on the conformation and exposure of sites interacting with cPLA₂ that become exposed in the native proteins only when forming membrane complexes after cell stimulation.

Discussion

The results of the present study are the first to demonstrate that in granulocytic cells, which contain abundant levels of NADPH oxidase, stimulation induces an immediate and transient recruitment of cPLA₂ to the plasma membranes, in addition to its already known translocation to the nuclear

membranes (Marshall et al., 2000). The translocation of cPLA₂ to the membrane fractions of stimulated neutrophils has been shown earlier by us and by others (Durstin et al., 1994; Hazan et al., 1997; Marshall et al., 2000). In addition, several studies performed in a variety of cells including platelets, fibroblasts, and keratinocytes (Kast et al., 1993; Schalkwijk et al., 1995; McNicol and Shibou, 1998; Kitatani et al., 2000) have reported translocation of cPLA₂ to membrane fractions. However, the present study demonstrates that cPLA₂ is localized in the plasma membranes upon stimulation. The absence of cPLA₂ translocation to the plasma membranes in granulocyte-like gp91^{phox}-deficient PLB-985 cells and the restoration of cPLA₂ translocation, after the expression of retroviral gp91^{phox} protein, indicate that NADPH oxidase is responsible for anchoring cPLA₂ to the plasma membranes. The translocation of cPLA₂ to the membranes occurred after assembly of the oxidase and coincided with the onset of superoxide production (Fig. 4). The affinity binding between GST-p47^{phox} or GST-p67^{phox} with cPLA₂ only in lysates of stimulated cells (Fig. 8) suggests a direct binding with cPLA₂ in its phosphorylated form. In earlier studies (Dana et al., 1998), we have shown that AA by itself and not its metabolites is required for activation of the assembled NADPH oxidase enzyme. The colocalization of both enzymes (the assembled NADPH oxidase and cPLA₂) in the same compartment and their direct binding during the onset of superoxide production provides a means by which AA released by cPLA₂ is able to activate the assembled NADPH oxidase. The precise mechanism by which AA regulates the NADPH oxidase in whole cells is not yet known. Several studies suggest that AA induces structural changes in NADPH oxidase components that may promote productive interaction between the different oxidase subunits, thereby enabling full oxidase activation or directly affecting the function of flavocytochrome b (Foubert et al., 2002). The 1:25 molar ratio between cPLA₂ to the assembled oxidase in the membranes of stimulated cells (Fig. 5) indicates that one copy of cPLA₂ is able to provide sufficient levels of AA for several copies of the NADPH oxidase found in its environment. Our current observations are most consistent with a model in which cPLA₂-generated AA might be a cofactor acting in the intact phagocytic cell to enhance the affinity of the assembled NADPH oxidase for NADPH (Rubinek and Levy, 1993) probably by induction of structural changes. cPLA₂ dissociates from the membranes before the oxidase cytosolic components (Fig. 4, D–F) and while the oxidase is still functioning (Fig. 4, G–I), suggesting that cPLA₂ is required for the activation of NADPH oxidase but not for maintaining its activity.

Recent studies have suggested that cPLA₂ has three functionally distinct domains: an NH₂-terminal C2 domain necessary for Ca²⁺-dependent phospholipid binding, a COOH-terminal Ca²⁺-independent catalytic region (Nalefski et al., 1994), and a putative pleckstrin homology domain within this region that is responsible for the interaction with phosphatidylinositol 4,5-bisphosphate (PtdIns[4,5]P₂; Mosior et al., 1998). Subcellular localization of the various C2 domains upon cytoplasmic [Ca²⁺] elevation has been shown to correlate with their phospholipid binding specificity. It has also been shown that the cPLA₂-C2 domain has specificity

to the phosphatidylcholine-rich nuclear envelope and ER (Gijon et al., 1999; Perisic et al., 1999; Hurley and Misra, 2000) and that aromatic and hydrophobic residues in the calcium binding loop of the cPLA₂-C2 domain are important for its lipid specificity (Stahelin et al., 2003b). In contrast, the PKCα-C2 domain has specificity to the phosphatidylserine-rich plasma membranes (Oancea and Meyer, 1998; Corbalan-Garcia et al., 1999) and ASn¹⁸⁹ plays a key role in this specificity (Stahelin et al., 2003b). Several studies have shown that cPLA₂ translocates from the cytosol to the nuclear membrane and to the ER by calcium ionophores or agonists such as histamine or IgE/antigen, which increase cytoplasmic [Ca²⁺] in a variety of cells (Peters-Golden and McNish, 1993; Glover et al., 1995; Schievella et al., 1995; Sierra-Honigmann et al., 1996). However, PMA, which does not induce an increase in cytoplasmic [Ca²⁺], caused activation and translocation of cPLA₂ (Hazan et al., 1997; Qiu et al., 1998), suggesting the existence of alternative pathways that induce translocation of cPLA₂ not involving elevation of cytoplasmic [Ca²⁺], which is necessary for the C2 domain phospholipid binding. The results of our present study suggest that the cPLA₂-C2 domain does not participate in the translocation of cPLA₂ to the plasma membranes, since cPLA₂ translocation is inconsistent with its C2 domain phospholipid binding specificity. In addition, cPLA₂ translocation to the cell periphery did not occur in the absence of a functional NADPH oxidase (Fig. 7), and it can be induced with PMA (Fig. 3 and Fig. 6 A), which does not cause elevation of cytoplasmic [Ca²⁺]. However, we cannot rule out the possibility that the binding of cPLA₂ to the assembled oxidase is mediated through the cPLA₂-C2 domain. Similar to our observation that the C2 domain-containing cPLA₂ binds NADPH oxidase, a recent study (McAdara Berkowitz et al., 2001) has shown that C2 domain-containing protein JFC1, which is restricted to the plasma membranes/secretory vesicles, binds p67^{phox} without affecting the interaction between p47^{phox} and p67^{phox}. High levels of PtdIns(4,5)P₂ have been detected in neutrophil plasma membranes upon stimulation (Botelho et al., 2000; Martin, 2001), and PtdIns(4,5)P₂ has been shown to display a particularly dramatic effect on the activity of cPLA₂ (Mosior et al., 1998). However, it seems that the cPLA₂ pleckstrin homology domain by itself does not play a critical role in targeting cPLA₂ to the plasma membranes, since in the absence of the functional oxidase cPLA₂ did not translocate to this compartment, as shown in gp91^{phox}-deficient granulocytic PLB-985 cells (Fig. 7). Thus, the assembled NADPH oxidase appears to be the major determinant in directing cPLA₂ to the plasma membranes, although the interaction sites among cPLA₂, NADPH oxidase, and the plasma membranes are not yet defined.

Eicosanoid generation has been shown to be regulated in part by perinuclear envelope localization or translocation of individual enzymes of leukotriene and prostaglandin biosynthesis (Ueno et al., 2001). The perinuclear translocation of cPLA₂, shown in a variety of cells, is in agreement with its role in leukotriene and prostaglandin formation. Similarly, our present study demonstrates a correlation between the kinetics of cPLA₂ translocation to nuclear membranes and PGE₂ production in stimulated granulocyte-like PLB-985 cells (Fig. 6, C and D). The differences in the kinetics of su-

peroxide production and PGE₂ formation in PLB-985 cells differentiated with DMSO enabled us to efficiently follow the distribution of cPLA₂ during stimulation, from early translocation to the cell periphery to later translocation to the nuclear envelope. Thus, the mechanism which permits the participation of cPLA₂ in two different processes in the same cell (regulation of NADPH oxidase and eicosanoid production) is controlled by localization of the enzyme in different subcellular compartments.

In conclusion, the use of combined biochemical and microscopical approaches enhanced our ability to gain better insight into cellular processes and to clearly demonstrate that upon activation of peripheral blood neutrophils and granulocyte-like PLB-985 cells, both of which contain abundant levels of NADPH oxidase, cPLA₂ translocates to the plasma membranes where it binds the assembled oxidase complex and releases AA, which promotes oxidase activity. The absence of cPLA₂ in the cell periphery of stimulated granulocyte-like gp91^{phox}-deficient PLB-985 cells and its translocation after the expression of retroviral gp91^{phox} protein in these cells indicate that cPLA₂ is anchored to the plasma membranes by the assembled oxidase. The physical association between these two enzymes enables the regulation of NADPH oxidase by cPLA₂-generating AA. The novel dual subcellular localization of cPLA₂ in different compartments, first in the plasma membranes and then in the nucleus, provides a molecular mechanism for the participation of cPLA₂ in different processes in the same cells. The exact binding sites between these two enzymes and the target for AA action are currently under investigation.

Materials and methods

Neutrophil purification

Neutrophils were separated by Ficoll/Hypaque centrifugation, dextran sedimentation, and hypotonic lysis of erythrocytes (Dana et al., 1998).

Cell culture and differentiation

PLB-985 and gp91^{phox}-deficient PLB-985 cells lacking the expression of normal gp91^{phox} (provided by M.C. Dinauer, James Whitcomb Riley Hospital for Children, Indianapolis, IN) were grown in stationary suspension culture in RPMI-1640 as described earlier (Dana et al., 1998). The optimal concentration of 1.25% of DMSO was added to 2 × 10⁵ PLB-985 cells/ml at their logarithm growth phase to induce differentiation toward the granulocyte phenotype. Mac-1 antigen determination was detected by indirect immunofluorescence as described previously (Hazav et al., 1989).

Isolation of membrane and cytosol fractions

Neutrophils were suspended at 10⁷ cells/ml in phosphate buffer saline and treated with 5 mM diisopropylfluorophosphate for 30 min at room temperature before stimulation. Membrane and cytosol fractions were prepared as described previously (Levy et al., 1990).

Immunoprecipitation

Goat antiserum raised against recombinant p47^{phox} or p67^{phox} (Leto et al., 1991) or rabbit antiserum raised against cPLA₂ (Hazan et al., 1997) was added to 3 × 10⁷ membranes or cytosol cell equivalents in 500 μl solubilization buffer (150 mM NaCl, 5 mM EDTA, 10 mM Tris, pH 7.5, 1% sodium deoxycholate, and 1% NP-40), and they were incubated on ice overnight. The extracts were brought to a volume of 1 ml in solubilization buffer containing 30 μl of 50% slurry of recombinant protein G-sepharose. The samples were tumbled end-over-end for 1 h and washed twice with 1 ml solubilization buffer containing 20% (wt/vol) sucrose and 0.15% (wt/vol) BSA and twice with 1 ml solubilization buffer containing 20% sucrose. The samples were boiled in SDS sample buffer and electrophoresed on a 10 or 7% SDS-PAGE. For detection of protein translocation, cell

membranes (2 × 10⁶ cell equivalent) were separated by SDS-PAGE electrophoresis. The resolved proteins were electrophoretically transferred to nitrocellulose, and the detection of cPLA₂ or the oxidase components was analyzed as described previously (Hazan et al., 1997).

Subcellular fractionation

Subcellular fractionation was performed as described by others (Kjeldsen et al., 1999). Neutrophils (5 × 10⁹) treated with diisopropylfluorophosphate were suspended in relaxation buffer (as described earlier; Levy et al., 1990) and disrupted by nitrogen cavitation at 400 pounds per square inch. Nuclei and unbroken cells were pelleted by centrifugation at 500 g for 10 min at 4°C. The supernatant was decanted and loaded onto a precooled discontinuous density gradient Percoll, and 10× concentrated relaxation buffer and distilled water were mixed to give solutions of densities of 1.05, 1.09, and 1.12 g/ml. The gradients were centrifuged at 32,800 g for 35 min at 4°C using a fixed angle Beckman Coulter JA20 rotor. Four visible bands were collected, and the markers for azurophil granules (α), specific granules (β), and plasma membranes (γ) were analyzed as described previously (Kaufman et al., 1996).

Immunofluorescence microscopy

Preparation of labeled cells was done as described by others (Bingham et al., 1999) with some modification. Cells were adhered on coverslips for 30 min at 37°C. The cells were stimulated with various agonists for the desired duration and fixed with 3% (wt/vol) formaldehyde. The first antibodies against cPLA₂, gp91^{phox}, and lamin B (Santa Cruz Biotechnology) dissolved in PBS containing 0.2% saponin were added for 1 h at room temperature. cy2- or cy3-conjugated antibodies (Jackson ImmunoResearch Laboratories) were used as secondary antibodies. The fluorescence was visualized using a four channel Zeiss LSM 510 laser scanning confocal microscope (Carl Zeiss MicroImaging, Inc.). The LSM 510 software was used for imaging cPLA₂, gp91^{phox}, and lamin B localization.

Retroviral transduction of gp91^{phox}-deficient PLB-985 cells

Retroviral gp91^{phox} was expressed in gp91^{phox}-deficient PLB-985 cells (Zhen et al., 1993) as done in our previous study (Pessach et al., 2001).

Affinity-binding assay

The GST fusion proteins were affinity purified on glutathione-sepharose as described previously (Leto et al., 1994). 50 μl (1 μg) of GST-p47^{phox} or GST-p67^{phox} bound to glutathione-sepharose beads was added to lysates of 5 × 10⁷ resting or stimulated neutrophils prepared as described before (Hazan-Halevy et al., 2000) and was tumbled end-over-end for 2 h at 4°C. Bound proteins were washed three times in 15 vol of ice cold 100 mM KCl, 3 mM NaCl, 3.5 mM MgCl₂, 0.15 mM PMSF, and 10 mM Pipes (pH 7.5), eluted with 2% SDS, and analyzed by SDS-PAGE followed by immunoblotting with the appropriate antibodies.

Superoxide anion measurements

The production of superoxide anion (O₂⁻) by intact cells was measured as the superoxide dismutase inhibitable reduction of ferricytochrome c (Dana et al., 1998).

Preparation of nuclei

Nuclei were separated from granulocyte-like PLB cells (2 × 10⁷ cells) before and after stimulation as described by others in neutrophils (Surette et al., 1998). Stimulated cells were pelleted and resuspended in 600 μl of ice-cold NP-40 lysis buffer (0.1% NP-40, 10 mM Tris-HCl, pH 7.4, 10 mM NaCl, 3 mM MgCl₂, 1 mM EDTA, 10 μg/ml leupeptin, 10 μg/ml aprotinin, and 1 mM PMSF). The cells were vortexed for 15 s, kept on ice for 5 min, and centrifuged at 300 g for 10 min at 4°C. The resulting pellets (the nuclei containing fractions) were then immediately solubilized in electrophoresis sample buffer and processed for SDS-PAGE and immunoblot determination of cPLA₂ and the nuclear lamin B. Nuclear integrity was verified directly by light microscopy, which also revealed that intact cells were rarely observed in nuclei-containing fraction (<2%).

PGE₂ determination

PGE₂ levels were determined in the supernatant of stimulated cells by RIA using commercial kits (NEN Life Science Products). The samples were immediately stored at -70°C and analyzed during 1 wk of the experiments.

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